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## NEW APPLICATION TRANSMITTAL - UTILITY

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**Inventor(s):** JOHN E. EDWARDS, JR., SCOTT G. FILLER, JIM CUTLER, DONALD C. SHEPPARD, ASHRAF S. IBRAHIM; AND YUE FU

**Title:** PHARMACEUTICAL COMPOSITIONS AND METHODS TO VACCINATE AGAINST DISSEMINATED CANDIDIASIS

**I. PAPERS ENCLOSED HEREWITH FOR FILING UNDER 37 CFR § 1.53(b):**

23 Page(s) of Written Description  
2 Page(s) Claims  
1 Page(s) Abstract  
14 Sheets of Drawings  Informal  Formal

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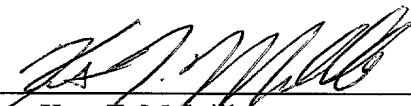
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Dated: November 18, 2000

**PHARMACEUTICAL COMPOSITIONS AND METHODS**  
**TO VACCINATE AGAINST DISSEMINATED CANDIDIASIS**

**RELATED INFORMATION**

5        This application claims priority from Provisional Application Serial No.: 60/166,663 filed November 19, 1999. This invention was made with Government support under Public Health Service grants PO-1AI-37194, RO1AI-19990, and MO1 RR0425. The Government has certain rights in this invention.

**FIELD OF INVENTION**

10      This invention relates to *Candida albicans* surface adhesin proteins, to antibodies resulting from an immune response to vaccination, and to methods for the prevention and/or treatment of candidiasis.

**BACKGROUND OF INVENTION**

15      There has been a dramatic increase in the incidence of nosocomial infections caused by *Candida* species in recent years. The incidence of hematogenously disseminated candidal infections increased 11-fold from 1980 to 1989. This increasing incidence has continued into the 1990s. Infections by *Candida* species are now the fourth most common cause of nosocomial septicemia, are equal to that of *Escherichia coli*, and surpass the incidence caused by *Klebsiella* species.  
20 Furthermore, *Candida* species are the most common cause of deep-seated fungal infections in patients who have extensive burns. Up to 11% of individuals undergoing bone marrow transplantation and 13% of those having an orthotopic liver transplant will develop an invasive candidal infection.

*Candida albicans*, the major pathogen in this genus, can switch between two morphologies: the blastospore (budding yeast) and filamentous (hyphae and pseudohyphae) phases. *Candida* mutants that are defective in genes regulating filamentation are reported to have reduced virulence in animal models. This reduced virulence suggests that the ability to change from a blastospore to a 5 filament is a key virulence factor of *C. albicans*. To date, no essential effectors of these filamentation pathways have been identified in *C. albicans*. See Caesar-TonThat, T.C. and J.E. Cutler, "A monoclonal antibody to *Candida albicans* enhances mouse neutrophil candidacidal activity." Infect. Immun. 65:5354-5357, 1997.

The identification of effectors in the regulatory pathways of the organism that contribute to 10 virulence offers the opportunity for therapeutic intervention with methods or compositions that are superior to existing antifungal agents. The identification of cell surface proteins that effect a regulatory pathway involved in virulence is particularly promising because characterization of the protein enable immunotherapeutic techniques that are superior to existing antifungal agents when fighting a candidal infection.

15 The virulence of *Candida albicans* is regulated by several putative virulence factors of which adherence to host constituents and the ability to transform from yeast-to-hyphae are among the most critical in determining pathogenicity. While potent antifungal agents exist that are microbicidal for *Candida*, the attributable mortality of candidemia is approximately 38%, even with treatment with potent anti-fungal agents such as amphotericin B. Also, existing agents 20 such as amphotericin B tend to exhibit undesirable toxicity. Although additional antifungals may be developed that are less toxic than amphotericin B, it is unlikely that agents will be developed that are more potent. Therefore, either passive or active immunotherapy to treat or prevent disseminated candidiasis is a promising alternative to standard antifungal therapy.

## SUMMARY OF INVENTION

The present invention utilizes the gene product of *C. albicans* agglutinin like sequence ALS1 as a vaccine to treat, prevent, or alleviate disseminated candidiasis. The invention takes advantage of the role of the ALS1 gene product in the adherence of the *C. albicans* to endothelial and epithelial cells and the susceptibility of the ALS1-expressed surface protein for use as a vaccine to retard the pathogenesis of the organism.

Pursuant to this invention, the ALS1 gene encodes a surface adhesin that is selected as the target of an immunotherapeutic strategy against *Candida Albicans*. A demonstration that the expression product of the ALS1 gene, the ALS1p protein, has structural characteristics typical of surface proteins and is, in fact, expressed on the cell surface of *C. albicans* is a critical criterion for proteins that act as adhesins to host tissues. In this case, ALS1p has a signal peptide at the N-terminus, a glycosylphosphatidylinosine (GPI) anchorage sequence in the C-terminus, and a central region comprising repeats rich in threonine and serine. Also, the ALS1 protein has many N-, and O-glycosylation sites, typical of proteins that are expressed on the cell surface. Indirect immunofluorescence using a monoclonal antibody directed against the N-terminus of Als1p revealed that Als1p is expressed during the log phase of blastospores. This expression of Als1p is increased during hyphal formation and is localized to the junction where the hyphal element extends from the blastospores as indicated by the diffused surface staining. Furthermore, this monoclonal antibody blocked the enhanced adherence of *C. albicans* overexpression mutant to endothelial cells, thereby establishing the principle for immunotherapy applications using Als1p.

Additional evidence that Als1p is a surface adhesin protein is based on data showing that antibodies that bind to the surface of *C. albicans* also bind to the surface of *S. cerevisiae* transformed

with ALS1, but not with empty plasmid. The ALS1 protein also shares significant homology with the alpha-agglutinin of *S. cerevisiae*, which is expressed on the cell surface and mediates the binding of mating type alpha cells to mating type a cells. Moreover, expression of the ALS1 gene in *S. cerevisiae* increases the adherence of this organism to endothelial cells by approximately 100-fold.

5 Because the ALS1 gene appears to encode a functional adhesin in *S cerevisiae*, it is certain that it also encodes a functional adhesin in *C. albicans*. The *ALS1* gene was originally isolated by Hoyer et al. without a known function. Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi. 1995.

*Candida albicans* *ALSI*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. 15:39-54. (See also US Patents 5,668,263 and 5,817,466.)

10 Thus, according to one aspect of the invention we provide an ALS1 surface adhesin protein, designated Als1p, or a fragment conjugate or analogue thereof, having useful properties when formulated in a pharmaceutical composition and administered as a vaccine. Als1p or functional analogues conjugates or derivatives thereof, is preferably obtained from *Candida albicans*. However, similar adhesin molecules or analogues or derivatives thereof may be of candidal origin and may be obtainable, for example, from strains belonging to the genera

15 *Candida*, for example *Candida parapsilosis*, *Candida krusei*, and *Candida tropicalis*. A surface adhesin protein according to the invention may be obtained in purified form, and thus, according to a preferred embodiment of the invention a substantially pure ALS1 *Candida albicans* surface adhesin protein, or functional analogue conjugates or derivative thereof, is formulated as a

20 vaccine to cause an immune response in a patient to block adhesion of the organism to the endothelial cells.

An analogue or derivative of the surface adhesion protein according to the invention may be identified and further characterized by the criteria described herein for the ALS1 gene and

gene product. For example, a null mutant of the analogue or derivative would share markedly reduced adhesion to endothelial cells compared to controls. Similarly, over-expression of the analogue or derivative in an appropriate model would show an increased adherence to endothelial cells compared to controls and would be confirmed as a cell surface adhesin in 5 accord with the criteria described above. Also, antisera to the analogue or derivative would cross-react with anti-ALS1 antibodies and would also exhibit increased survival times when administered in a mouse model of disseminated *candidiasis* as disclosed herein.

The present invention also provides an immunotherapeutic strategy against *Candida* infection at the level of binding to the vascular endothelial cells and through a downstream effector of the filamentation regulatory pathway. An immunotherapeutic strategy is uniquely advantageous in this context because: (i) the morbidity and mortality associated with hematogenously disseminated candidiasis remains unacceptably high, even with currently available antifungal therapy; (ii) a rising incidence of antifungal resistance is associated with the increasing use of antifungal agents, iii) the population of patients at risk for serious *Candida* infections is well-defined and very large, and includes post-operative patients, transplant patients, cancer patients and low birth weight infants; and iv) a high percentage of the patients who develop serious *Candida* infections are not neutropenic, and thus may respond to a vaccine. For these reasons, *Candida* is the most attractive fungal target for either passive or active immunotherapy.

Having determined the immunotherapeutic potential of Als1p according to this invention, 20 this protein and conjugates analogues, or derivative molecules thereof may be used in treatment and/or prevention of candidal infections. Standard immunological techniques may be employed with the adhesion protein molecule, and its analogues, conjugates, or derivatives, to use the

molecule as an immunogen in a pharmaceutically acceptable composition administered as a vaccine. For the purposes of this invention, “pharmaceutical” or “pharmaceutically acceptable” compositions are formulated by known techniques to be non-toxic and, when desired, used with carriers or additives that are approved for administration to humans in, for example, intravenous, 5 intramuscular, intraperitoneal or sub-cutaneous injection. Such compositions may include buffers, salts or other solvents known to these skilled in the art to preserve the activity of the vaccine in solution.

With respect to the molecule used as the immunogen pursuant to the present invention, those of skill in the art will recognize that the Als1p molecule may be truncated or fragmented without losing the essential qualities as a vaccine. For example, Als1p may be truncated to yield 10 an N-terminal fragment by truncation from the C-terminal end with preservation of the functional properties described above. Likewise, C-terminal fragments may be created by truncation from the N-terminal end with preservation of the functional properties described above. Other modifications in accord with the foregoing rationale may be made pursuant to this 15 invention to create other Als1p analogs or derivatives, to achieve the benefits described herein with the native protein.

The goal of the immunotherapy provided by this invention to interfere with regulation of filamentation, to block adherence of the organism to host constituents, and to enhance clearance 20 of the organism by immunoefector cells. Since endothelial cells cover the majority of the vasculature, strategies to block the adherence of the organism to endothelial cells using antibodies are a preferred embodiment of the present invention and such adherence blocking strategies include active or passive immunotherapy directed against the candidal adhesin(s) disclosed herein. Thus, for example, any suitable host may be injected with protein and the

serum collected to yield the desired anti-adhesin antibody after appropriate purification and/or concentration. Prior to injection, the adhesin protein may be formulated in a suitable vehicle, preferably a known immunostimulant such as a polysaccharide. Thus, according to a further aspect of the invention we provide a pharmaceutical composition comprising a candidal adhesin 5 protein together with one or more pharmaceutically acceptable excipients in a formulation for use as a vaccine.

The method of the invention is ameliorating and/or preventing candidal infection by blocking the adherence of *C. albicans* to the endothelial cells of a host constituent. Thus, according to one aspect of the invention, a pharmaceutical composition comprising an ALS1 10 adhesin protein derivative, analogue, or conjugate is formulated as a vaccine in a pharmaceutical composition containing a biocompatible carrier for injection or infusion and is administered to a patient. Also, direct administration of antiserum raised against ALS1 protein may be used to block the adherence of *C. albicans* to a mammalian host constituent. Antiserum against adhesin 15 protein can be obtained by known techniques, Kohler and Milstein, Nature 256: 495-499 (1975), and may be humanized to reduce antigenicity, see USP 5,693,762, or produced in transgenic mice leaving an unarranged human immunoglobulin gene, see USP 5,877,397.

A still further use of the invention, for example, is using the ALS1 adhesin protein to develop vaccine strategies for the prevention and/or amelioration of candidal infections. Thus, according to one aspect of the invention, for example, standard immunology techniques may be 20 employed to construct a multi-component vaccine strategy that may enhance and/or elicit immune response from a host constituent to block adherence of *C. albicans*.

A still further use of the invention, for example, is developing DNA vaccine strategies. Thus, according to one aspect of the invention, for example, the ALS1 polynucleotide encoding

Als1p on a fragment thereof is administered according to a protocol designed to yield an immune response to the gene product. See e.g., Felgner USP 5,703,055.

A still further use of the invention, for example, is developing combination vaccine strategies. Thus, according to one aspect of the invention, for example, anti-ALS antibodies may 5 be used with antibodies in treating and/or preventing candidal infections. See USP 5,578,309.

#### DESCRIPTION OF THE FIGURES

Figure 1A, 1B show the mediation of Als1p adherence of *C. albicans* to human umbilical vein endothelial cells. Values represent the mean  $\pm$  SD of at least three independent experiments, each performed in triplicate. (A) Endothelial cell adherence of *ALS1/als2*, *als1/als1* and *ALS1*-complemented mutants and wild-type CAI12 (30) (B) Endothelial cell adherence of *P<sub>ADH1</sub>-ALS1* mutant that overexpresses *ALS1*, compared to wild type *C. albicans*. Statistical treatment was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction. \**P*<0.001 for all comparisons.

Figure 2A-D shows the cell surface localization of Als1p on filaments of *C. albicans* by indirect immunofluorescence. Filamentation of *C. albicans* was induced by incubating yeast cells in RPMI 1640 medium with glutamine for 1.5 hours at 37°C. Als1p was detected by incubating organisms first with anti-Als1p mouse mAb followed by FITC-labeled goat anti-mouse IgG. *C. albicans* cell surface was also stained with anti-*C. albicans* polyclonal Ab conjugated with Alexa 594 (Molecular Probes, Eugene, OR). Areas with yellow staining represent Als1p localization. (A) *C. albicans* wild-type. (B) *als1/als1* mutant strain. (C) *als1/als1* complemented with wild type *ALS1* (D) *P<sub>ADH1</sub>-ALS1* overexpression mutant.

Figure 3A, 3B show the mediation of Als1p on *C. albicans* filamentation on solid medium. *C. albicans* blastospores were spotted on Lee's agar plates and incubated at 37°C for 4 days (A) or 3 days (B).

Figure 4A, 4B show the control of *ALS1* expression and the mediation of *C. albicans* filamentation by the *EFG1* filamentation regulatory pathway. (A) Northern blot analysis showing expression of *ALS1* in (i) mutants deficient in different filamentation regulatory pathways. (ii) *efg1/efg1* mutant complemented with either *EFG1* or *P<sub>ADH1</sub>-ALS1*. Total RNA was extracted from cells grown in RPMI 1640 + glutamine medium at 37°C for 90 minutes to induce filamentation. Blots were probed with *ALS1* and *TEF1*. (B) Photomicrographs of the *efg1/efg1* mutant and *efg1/efg1* mutant complemented with *P<sub>ADH1</sub>-ALS1* grown on Lee's agar plates at 37°C. for 4 days.

Figure 5A, 5B show the reduction of virulence in the mouse model of hematogenously disseminated candidiasis by (A) Male Balb/C mice ( $n = 30$  for each yeast strain) were injected with stationary phase blastospores ( $10^6$  per mouse in 0.5 ml of PBS). Curves are the compiled results of three replicate experiments ( $n = 30$  mice for each strain). The doubling times of all strains, grown in YPD at 30°C, ranged between 1.29 to 1.52 hours and were not statistically different from each other. Southern blot analysis of total chromosomal DNA was used to match the identity of the genotype of *C. albicans* strains retrieved from infected organs with those of *C. albicans* strains used to infect the mice. Statistical analysis was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction. \* $P < 0.002$  for the *als1/als1* mutant versus each of the other strains. (B) Histological micrographs of kidneys infected with *C. albicans* wild-type, homozygous *als1 null* mutant, or heterozygous *ALS1* complemented mutant. Kidney samples were retrieved 28 hours (a) or 40 (b) hours post

infection, fixed in paraformaldehyde and sections were stained with silver (magnification, X400). Arrows denote *C. albicans* cells.

Figure 6 shows the prophylactic effect of anti-ALS antibody against disseminated candidiasis as a function of surviving animals over a 30-day period for animals infused with anti-

5 Als1p polyserum.

Figure 7 is the polypeptide sequence of Als1p.

### DETAILED DESCRIPTION OF THE INVENTION

The nature of the pathogenesis of *C. albicans* by adherence to endothelial cells is discussed in USP 5,578,309 which is specifically incorporated herein by reference in its entirety. For a description of the ALS1 gene and characteristics thereof, including the characterization of the gene product as an adhesin, see Fu, Y., S. G. Filler, B. J. Spellberg, W. Fonzi, A. S. Ibrahim, T. Kanbe, M. A. Ghannoum, and J. E. J. Edwards . 1998. Cloning and characterization of CAD I/AAF1, a gene from *Candida albicans* that induces adherence to endothelial cells after expression in *Saccharomyces cerevisiae*. Infect. Immun. 66:2078-2084; Fu, Y., G. Rieg, W. A. Forizi, P. H. Belanger, J. E. J. Edwards, and S. G. Filler. 1998. Expression of the *Candida albicans* gene *ALSI* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. Infect. Immun. 66:1783-1786; Hoyer, L.L. 1997. The ALS gene family of *Candida albicans*. International Society for Human and Animal Mycology Salsimorge, Italy:(Abstract); Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi. 1995. *Candida albicans* *ALSI*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. 15:39-54.

The following Examples illustrate the immunotherapeutic utility of the ALS1 adhesin as the basis for preventive measures or treatment of disseminated candidiasis. Example 1 describes the preparation of an ALS1 null mutant and a strain of *C. albicans* characterized by over-expression of ALS1 to confirm the mediation of adherence to endothelial cells. Example 2 5 describes the localization of Als1p and the implication of the efg filamentation regulatory pathway. Example 3 describes the purification of ALS1 adhesin protein. Example 4 describes the preparation of rabbit polyclonal antibodies raised against the ALS1 surface adhesin protein to be used to demonstrate the blocking of the surface adhesin protein. Example 5, describes the blocking of adherence in vivo, using polyclonal antibodies raised against the ALS1 surface 10 adhesion protein as described herein according to the invention to protect against disseminated candidiasis in a mouse model.

#### **EXAMPLE 1 – Als1 Mediates Adherence of *C. albicans* to Endothelial Cells**

The URA blaster technique was used to construct a null mutant of *C. albicans* that lacks expression of the Als1p. The *als1/als1* mutant was constructed in *C. albicans* strain CAI4 using a modification of the Ura-blaster methodology [W. A. Fonzi and M. Y. Irwin, *Genetics* 134, 717 15 (1993)] as follows: Two separate *als1-hisG-URA3-hisG-als1* constructs were utilized to disrupt the two different alleles of the gene. A 4.9 kb *ALS1* coding sequence was generated with high fidelity PCR (Boehringer Mannheim, Indianapolis, IN) using the primers: 5'-  
20 CCGCTCGAGATGCTTACAACAATTACATTGTTA-3' and 5'-  
CCGCTCGAGTCACTAAATGAACAAAGGACAATA3'. Next, the PCR fragment was cloned into pGEM-T vector (Promega, Madison, WI), thus obtaining pGEM-T-*ALS1*. The *hisG-URA3-hisG* construct was released from pMG-7 by digestion with *KpnI* and *Hind3* and used to replace

the portion of *ALS1* released by *Kpn1* and *Hind3* digestion of pGEM-T-*ALS1*. The final *als1-hisG-URA3-hisG-als1* construct was released from the plasmid by digestion with *Xhol* and used to disrupt the first allele of *ALS1* by transformation of strain CAI-4.

A second *als1-hisG-URA3-hisG-als1* construct was generated in two steps. First, a *Bgl2-Hind3 hisG-URA3-hisG* fragment of pMB7 was cloned into the *BamH1-Hind3* sites of pUC19, thereby generating pYC2. PYC2 was then digested with *Hind3*, partially filled in with dATP and dGTP using T4 DNA polymerase, and then digested with *Sma1* to produce a new *hisG-URA3-hisG* fragment. Second, to generate *ALS1* complementary flanking regions, pGEM-T-*ALS1* was digested with *Xba1* and then partially filled in with dCTP and dTTP. This fragment was digested with *HpaI* to delete the central portion of *ALS1* and then ligated to the *hisG-URA3-hisG* fragment generating pYC3. This plasmid was then digested by *Xhol* to release a construct that was used to disrupt the second allele of the *ALS1*. Growth curves were done throughout the experiment to ensure that the generated mutations had no effect on growth rates. All integrations were confirmed by Southern blot analysis using a 0.9kb *ALS1* specific probe generated by digestion of pYF5 with *Xba1* and *HindIII*.

The null mutant was compared to *C. albicans* CAI-12 (a URA + revertant strain) for its ability to adhere in vitro to human umbilical vein endothelial cells. For adherence studies, yeast cells from YPD (2% glucose, 2% peptone, and 1% yeast extract) overnight culture, were grown in RPMI with glutamine at 25°C for 1 hour to induce Als1p expression.  $3 \times 10^2$  organisms in Hanks balanced salt solution (HBSS) (Irvine Scientific, Irvine, CA) were added to each well of endothelial cells, after which the plate was incubated at 37°C for 30 minutes. The inoculum size was confirmed by quantitative culturing in YPD agar. At the end of incubation period, the nonadherent organisms were aspirated and the endothelial cell monolayers were rinsed twice

with HBSS in a standardized manner. The wells were over laid with YPD agar and the number of adherent organisms were determined by colony counting. Statistical treatment was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction.  $P<0.001$ .

Referring to Figure 1, a comparison of the ALS1/ALS1 and als1/als1 strain showed that the ALS1 null mutant was 35% less adherent to endothelial cells than *C. albicans* CAI-12. To reduce background adherence, the adherence of the wild-type strain grown under non-ALS1 expressing conditions was compared with a mutant autonomously expressing A1s1p. This mutant was constructed by integrating a third copy of ALS1 under the control of the constitutive ADH1 promoter into the wild-type *C. albicans*. To achieve constitutive expression of the ALS1 in *C. albicans*, a blunt-ended PCR generated *URA3* gene is ligated into a blunt-edged *Bg*12 site of pOCUS-2 vector (Novagen, Madison, WI), yielding pOU-2. A 2.4 kb *Not*1-*Stu* fragment, which contained *C. albicans* alcohol dehydrogenase gene (ADH1) promoter and terminator (isolated from pLH-ADHpt, and kindly provided by A. Brown, Aberdeen, UK), was cloned into pOU-2 after digestion with *Not*1 and *Stu*. The new plasmid, named pOAU-3 had only one *Bg*12 site between the *ADH1* promoter and terminator. *ALS1* coding sequence flanked by *Bam*H1 restriction enzyme sites was generated by high fidelity PCR using pYF-5 as a template and the following primers: 5'-CGGGATCCAGATGCTTCA-ACAATTACATTG-3' and 5'-CGGGATCCTCACTAAATGAACAAAGGACAATA-3'. This PCR fragment was digested with *Bam*H1 and then cloned into the compatible *Bg*12 site of pOAU-3 to generate pAU-1. Finally, pAU-1 was linearized by *Xba*I prior to transforming *C. albicans* CAI-4. The site-directed integration was confirmed by Southern Blot analysis. Referring to Figure 1B, overexpressing *ALS1* in this *P<sub>ADH1</sub>-ALS1* strain resulted in a 76% increase in adherence to endothelial cells,

compared to the wild-type *C. albicans*. In comparing endothelial cell adherence of the wild-type to that of the overexpressing mutant, yeast cells were grown overnight in YPD at 25°C (non-inducing condition of Als1p). Als1p expression was not induced to reduce the background adherence of the wild-type, thus magnifying the role of Als1p in adherence through *P<sub>ADH1</sub>-ALS1* hybrid gene. The adherence assay was carried out as described above. Statistical treatment was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction. *P*<0.001.

A monoclonal anti-Als1p murine IgG antibody was raised against a purified and truncated N-terminus of Als1p (amino acid #17 to #432) expressed using Clontech YEXpress (™) Yeast Expression System (Palo Alto, CA). The adherence blocking capability of these monoclonal anti-Als1p antibodies was assessed by incubating *C. albicans* cells with either anti-Als1 antibodies or mouse IgG (Sigma, St. Louis, MO) at a 1:50 dilution. After which the yeast cells were used in the adherence assay as described above. Statistical treatment was obtained by Wilcoxon rank sum test and corrected for multiple comparisons with the Bonferroni correction. *P*<0.001. The results revealed that the adherence of the *P<sub>ADH1</sub>-ALS1* strain was reduced from 26.8%± 3.5% to 14.7%± 5.3%. Thus, the effects of ALS1 deletion and overexpression demonstrate that Als1p mediates adherence of *C. albicans* to endothelial cells.

## EXAMPLE 2 – Localization of Als1p

For Als1p to function as an adhesin, it must be located on the cell surface. The cell surface localization of Als1p was verified using indirect immunofluorescence with the anti-Als1p monoclonal antibody. Diffuse staining was detected on the surface of blastospores during exponential growth. This staining was undetectable on blastospores in the stationary phase.

Referring to Figure 2A, when blastospores were induced to produce filaments, intense staining was observed that localized exclusively to the base of the emerging filament. No immunofluorescence was observed with the *als1/als1* mutant, confirming the specificity of this antibody for Als1p. See Figure 2B. These results establish that Als1p is a cell surface protein.

5       The specific localization of Als1p to the blastospore-filament junction implicates Als1p in the filamentation process. To determine the mechanism, the filamentation phenotype of the *C. albicans* *ALS1* mutants was analyzed. Referring to Figure 3A, the *als1/als1* mutant failed to form filaments after a 4 day incubation on Lee's solid medium, while both the *ALS1/ALS1* AND *ALS1/als1* strains as well as the *ALS1*-complemented mutant produced abundant filaments at this time point. The *als1/als1* mutant was capable of forming filaments after longer periods of incubation. Furthermore, overexpressing *ALS1* augmented filamentation: the P<sub>ADH1</sub>-*ALS1* strain formed profuse filaments after a 3 day incubation, whereas the wild-type strain produced scant filaments at this time point. See Figure 3B. To further confirm the role of Als1p in filamentation, a negative control was provided using mutant similar to the *ALS1* overexpression mutant, except the coding sequence of the *ALS1* was inserted in the opposite orientation. The filamentation phenotype of the resulting strain was shown to be similar to that of the wild-type strain. The filament-inducing properties of Als1p are specific to cells grown on solid media, because all of the strains described above filamented comparably in liquid media. The data demonstrates that Als1p promotes filamentation and implicates *ALS1* expression in the regulation of filamentation control pathways. Northern blot analysis of *ALS1* expression in mutants with defects in each of these pathways, including *efg1/efg1*, *cph1/cph1*, *efg1/efg* *cph1/cph1*, *tup1/tup1*, and *cla4/cla4* mutants were performed. Referring to Figure 4A, mutants in which both alleles of *EFG1* had been disrupted failed to express *ALS1*. Introduction of a copy

of wild-type *EFG1* into the *efg1/efg1* mutant restored *ALS1* expression, though at a reduced level. See Figure 4B. Also, as seen in Figure 4A, none of the other filamentation regulatory mutations significantly altered *ALS1* expression (Fig. 4A). Thus, Efg1p is required for *ALS1* expression.

If Efg1p stimulates the expression of *ALS1*, which in turn induces filamentation, the expression of *ALS1* in the *efg1/efg1* strain should restore filamentation. A functional allele of *ALS1* under the control of the *ADH1* promoter was integrated into the *efg1/efg1* strain. To investigate the possibility that *ALS1* gene product might complement the filamentation defect in *efg1* null mutant, an Ura *efg1* null mutant was transformed with linearized pAU-1. Ura<sup>+</sup> clones were selected and integration of the third copy of *ALS1* was confirmed with PCR using the primers: 5'-CCGTTTATACCATCCAAATC-3' and 5'-CTACATCCTCCAATGATATAAC-3'. The resulting strain expressed *ALS1* autonomously and regained the ability to filament on Lee's agar. See Figures 4B and C. Therefore, Efg1p induces filamentation through activation of *ALS1* expression.

Because filamentation is a critical virulence factor in *C. albicans*, delineation of a pathway that regulates filamentation has important implications for pathogenicity. Prior to *ALS1*, no gene encoding a downstream effector of these regulatory pathways had been identified. Disruption of two other genes encoding cell surface proteins, *HWP1* AND *INT1*, results in mutants with filamentation defects. Although *HWP1* expression is also regulated by Efg1p, the autonomous expression of *HWP1* in the *efg1/efg1* mutant fails to restore filamentation. Therefore Hwp1p alone does not function as an effector of filamentation downstream of *EFG1*. Also, the regulatory elements controlling *INT1* expression are not known. Thus, Als1p is the first

cell-surface protein identified that functions as a downstream effector of filamentation, thereby suggesting a pivotal role for this protein in the virulence of *C. albicans*.

The contribution of Als1p to *C. albicans* virulence was tested in a model of hematogenously disseminated candidiasis, A.S. Ibrahim *et al.*, *Infect. Immun.* **63**, 1993 (1995).

5 Referring to Figure 5A, mice infected with the *als1/als1* null mutant survived significantly longer than mice infected with the *ALS1/ALS1* strain, the *ALS1/als1* mutant or the *ALS1*-complemented mutant. After 28 hours of infection, the kidneys of mice infected with the *als1/als1* mutant contained significantly fewer organisms ( $5.70 \pm 0.46 \log_{10}$  CFU/g) ( $P < 0.0006$  for both comparisons). No difference was detected in colony counts of organisms recovered from spleen, lungs, or liver of mice infected with either of the strains at any of the tested time points. These results indicate that Als1p is important for *C. albicans* growth and persistence in the kidney during the first 28 hours of infection. Referring to Figure 5B, examination of the kidneys of mice after 28 hours of infection revealed that the *als1/als1* mutant produced significantly shorter filaments and elicited a weaker inflammatory response than did either the wild-type or *ALS1*-complemented strains. However, by 40 hours of infection, the length of the filaments and the number of leukocytes surrounding them were similar for all three strains.

10 The filamentation defect of the *als1/als1* mutant seen on histopathology paralleled the in vitro filamentation assays on solid media. This mutant showed defective filamentation at early time points both in vivo and in vitro. This defect eventually resolved with prolonged 15 infection/incubation. These results suggest that a filamentation regulatory pathway that is independent of *ALS1* may become operative at later time points. The activation of this alternative filamentation pathway by 40 hours of infection is likely the reason why mice infected with the *als1/als1* mutant subsequently succumbed in the ensuing 2-3 days.

Collectively, these data demonstrate that *C. albicans* *ALS1* encodes a cell surface protein that mediates both adherence to endothelial cells and filamentation. Als1p is the only identified downstream effector of any known filamentation regulatory pathway in *C. albicans*.

Additionally, Als1p contributes to virulence in hematogenous candidal infection. The cell 5 surface location and dual functionality of Als1p make it an attractive target for both drug and immune-based therapies.

### **EXAMPLE 3 -- Purification of ALS1 Adhesin Protein**

The ALS1 protein synthesized by *E. coli* is adequate as an immunogen. However, 10 eukaryotic proteins synthesized by *E. coli* may not be functional due to improper folding or lack of glycosylation. Therefore, to determine if the ALS1 protein can block the adherence of *C. albicans* to endothelial cells, the protein is, preferably, purified from genetically engineered *C. albicans*. 15

PCR was used to amplify a fragment of *ALS1*, from nucleotides 52 to 1296. This 1246 bp fragment encompassed the N-terminus of the predicted ALS1 protein from the end of the signal peptide to the beginning of the tandem repeats. This region of *ALS1* was amplified because it likely encodes the binding site of the adhesin, based on its homology to the binding region of the *S. cerevisiae* *Agα1* gene product. In addition, this portion of the predicted ALS1 protein has few glycosylation sites and its size is appropriate for efficient expression in *E. coli*.

20 The fragment of *ALS1* was ligated into pQE32 to produce pINS5. In this plasmid, the protein is expressed under control of the *lac* promoter and it has a 6-hits tag fused to its N-terminus so that it can be affinity purified. We transformed *E. coli* with pINS5, grew it under inducing conditions (in the presence of IPTG), and then lysed the cells. The cell lysate was

passed through a Ni<sup>2+</sup> -agarose column to affinity purify the *ALS1*-6His fusion protein. This procedure yielded substantial amounts of *ALS1*-6His. The fusion protein was further purified by SDS-PAGE. The band containing the protein was excised from the gel so that polyclonal rabbit antiserum can be raised against it. It will be appreciated by one skilled in the art that the surface adhesin protein according to the invention may be prepared and purified by a variety of known processes without departing from the spirit of the present invention. The sequence of Als1p is listed in Figure 7.

#### EXAMPLE 4 -- Raising Polyclonal Antisera against ALS1 Protein

To determine whether antibodies against the ALS1 protein block the adherence of *Candida albicans* to endothelial and epithelial cells, and the selected host constituent in vitro, rabbits were inoculated with *S. cerevisiae* transformed with ALS1 protein. The immunization protocol used was the dose and schedule used by Hasenclever and Mitchell for production of antisera that identified the antigenic relationship among various species of *Candida*.

Hasenclever, H. F. and W. O. Mitchell. 1960. Antigenic relationships of *Torulopsis glabrata* and seven species of the genus *Candida*. J. Bacteriol. 79:677-681. Control antisera were also raised against *S. cerevisiae* transformed with the empty plasmid. All yeast cells were grown in galactose to induce expression of the ALS genes. Before being tested in the adherence experiments, the serum was heat-inactivated at 56 C to remove all complement activity.

Sera from immunized rabbits were absorbed with whole cells of *S. cerevisiae* transformed with empty plasmid to remove antibodies that are reactive with components of the yeast other than ALS1 protein. The titer of the antisera was determined by immunofluorescence using *S. cerevisiae* that express the ALS1 gene. FITC-labeled anti-rabbit antibodies were

purchased from commercial sources (Southern Biotechnology, Inc). Affinity-purified secondary antibodies were essential because many commercially available sera contain antibodies reactive with yeast glucan and mannan. The secondary antibodies were pretested using *Candida albicans* as well as *S. cerevisiae* transformed with the plasmid and were absorbed as needed to remove 5 any anti-*S. cerevisiae* or anti-*Candida* antibodies. Negative controls were 1) preimmune serum, 2) *S. cerevisiae* transformed with the empty plasmid, and 3) *S. cerevisiae* transformed with the ALS gene but grown under conditions that suppress expression of the ALS gene (glucose).

In addition to the above experiments, Western blotting was used to provide further confirmation that an antiserum binds specifically to the ALS protein against which it was raised. 10 *S. cerevisiae* transformed with the ALS1 were grown under inducing conditions and their plasma membranes were isolated by standard methods. Panaretou, B. and P. Piper. 1996. Isolation of yeast plasma membranes. p. 117-121. In I.H. Evans. (ed.), Yeast Protocols. Methods in Cell and Molecular Biology. Humana Press, Totowa, New Jersey. Plasma 15 membranes were also prepared from *S. cerevisiae* transformed with the empty plasmid and grown under identical conditions. The membrane proteins were separated by SDS-PAGE and then transferred to PVDF membrane by electroblotting. Harlow, E. and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press. After being blocked with nonfat milk, the blot was incubated with the ALS antiserum. The preabsorbed antiserum did not react with proteins extracted from *S. cerevisiae* containing empty plasmid. This 20 antiserum blocked the adherence of *S. cerevisiae* pYF5 ( a clone that expresses *Candida albicans* ALS1) to endothelial cells.

**EXAMPLE 5 -- Polyclonal Antibodies Against Specific ALS Proteins Prophylactically Protect Mice from Mucosal and Hematogenously Disseminated Candidal Infections.**

Having identified the antisera that block the adherence of a clone of *S. cerevisiae* transformed with an ALS1 ALS gene under the above conditions, these antisera were 5 demonstrated to protect mice from intravenous challenge with *Candida albicans*.

The antisera against the ALS proteins were first tested in the murine model of hematogenously disseminated candidiasis. Affinity-purified anti-ALS antibodies are effective in preventing adhesion of yeast cells to various substrates (see EXAMPLE 3). Affinity-purification is useful in this system because antibody doses can be accurately determined. Moreover, the unfractionated antisera will undoubtedly contain large amounts of antibody directed toward 10 antigens on the *S. cerevisiae* carrier cells. Many of these anti-*Saccharomyces* antibodies would likely bind to *C. albicans* and make interpretation of the results impossible. Additionally, it is quite possible that the procedure used to elute antibodies from *S. cerevisiae* that express the 15 ALS protein may also elute small amounts of yeast mannan or glucan that could have adjuvant-like activity. The immunoaffinity-purified antibodies are further purified before use. They may also be preabsorbed with mouse splenocytes.

Antibody doses may be administered to cover the range that brackets the levels of serum antibody that can be expected in most active immunization protocols and to cover the range of antibody doses that are typically used for passive immunization in murine models of candidiasis.

20 See Dromer, F., J. Charreire, A. Contrepose, C. Carbon, and P. Yeni. 1987, Protection of mice against experimental cryptococcosis by anti-Cryptococcus neoformans monoclonal antibody, Infect. Immun. 55:749-752; Han, Y. and J. E. Cutler. 1995, Antibody response that protects against disseminated candidiasis, Infect. Immun. 63:2714-2719; Mukherjee, J., M. D. Scharff,

and A. Casadevall. 1992, Protective murine monoclonal antibodies to *Cryptococcus neoformans*, Infect. Immun. 60:4534-4541; Sanford, J. E., D. M. Lupon, A. M. Schlageter, and T. R. Kozel. 1990, Passive immunization against *Cryptococcus neoformans* with an isotype-switch family of monoclonal antibodies reactive with cryptococcal polysaccharide, Infect. Immun. 58:1919-1923.

5 BALB/c mice (female, 7 week old, the NCI) were given anti-ALS that had been absorbed with mouse splenic cells by an intraperitoneal (i.p.) injection. Control mice received prebled serum that had been absorbed with mouse spenic cells, intact anti-ALS serum, or DPBS, respectively. For the pre-absorption, 2 ml of anti-ALS or prebled sera were mixed with  $100\mu\text{l}$  of mouse (BALB/c, 7 weeks old female, NCI) splenic cells (app.  $9 \times 10^6$  cells per ml) at room temperature for 20 minutes. The mixture was washed with warm sterile DPBS by centrifugation (@ 300 xg) for 3 minutes. This procedure was repeated three times. The volume of i.p. injection was 0.4 ml per mouse. Four hours later, the mice were challenged with *C. albicans* (strain CA-1;  $5 \times 10^5$  hydrophilic yeast cells per mouse) by i.v. injection. Then, their survival times were measured.

See Figure 6.

15 Previous studies have shown that antibodies administered via the intraperitoneal route are rapidly (within minutes) and almost completely transferred to the serum (Kozel and Casadevall, unpublished observations). As a control for effects of administering the antibody preparations, a parallel group of mice were treated with antibodies isolated from pre-immune serum that has been absorbed with *S. cerevisiae* transformed with the ALS gene. The survival time and numbers of yeast per gram of kidney were measured. Again, referring to Figure 6, mice infected 20 intravenously with  $10^6$  blastopores of ALS1 null mutant had a longer median survival time when compared to mice infected with *Candida albicans* CAI-12 or *Candida albicans* in which one allele of the ALS1 had been deleted ( $p=0.003$ ).

These results indicate that immunotherapeutic strategies using the ALS1 proteins as a vaccine have a protective prophylactic effect against disseminated candidiasis.

The particular examples set forth herein are instructional and should not be interpreted as limitations on the applications to which those of ordinary skill are able to apply this invention.

5 Modifications and other uses are available to those skilled in the art which are encompassed within the spirit and scope of the following claims.

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## CLAIMS

I claim:

1. A vaccine comprising:

5 a pharmaceutical composition comprising isolated and purified cell surface adhesin protein obtained from a *Candida* strain.

2. The vaccine of Claim 1, wherein the cell surface adhesin protein is *Candida albicans* ALS1 adhesion protein (Als1p).

3. The vaccine of Claim 2, wherein the protein is a fragment encompassing the N-terminus of Als1p and containing the adhesin binding site.

4. The vaccine of Claim 1, wherein said cell surface adhesin protein is derived from a Candidal strain selected from the group consisting of *Candida krusei*, *Candida tropicalis*, and *Candida parapsilosis*.

5. A method for treatment and prevention of disseminated candidiasis, comprising:  
15 administering a vaccine comprised of a pharmaceutically effective amount of an ALS1 adhesion protein of *Candida albicans* in an amount elicit to an immune response.

6. The method of Claim 5, wherein said step of administering vaccine comprises the step of using active immunization through administration of the *Candida albicans* ALS1 gene product.

20 7. The method of Claim 5, wherein said step of administering vaccine comprises passive immunization through administration of antiserum to ASL1 gene product.

8. The method of Claim 5, wherein said step of administering vaccine further comprises the step of using a combination of active immunotherapy with the ALS1 gene product and passive immunotherapy with antiserum to the ALS1 gene product.

**ABSTRACT:**

A *Candida albicans* bloodstream infections cause significant morbidity and mortality in hospitalized patients. Filament formation and adherence to host cells are critical virulence factors of *C. albicans*. Multiple filamentation regulatory pathways have been discovered, 5 however the downstream effectors of these regulatory pathways remain unknown. The cell surface protein, Als1p, is a downstream effector of the filamentation regulatory pathway and is regulated by Efg1p. Als1p mediates adherence to endothelial cells in vitro and is required for virulence. The blocking of adherence by the organism is described resulting from the use of a composition and method disclosed herein. Specifically, a pharmaceutical composition comprised of a gene product from the ALS1 gene family is administered as a vaccine to generate 10 an immune response capable of blocking adherence of the organism.

FIGURE 1A

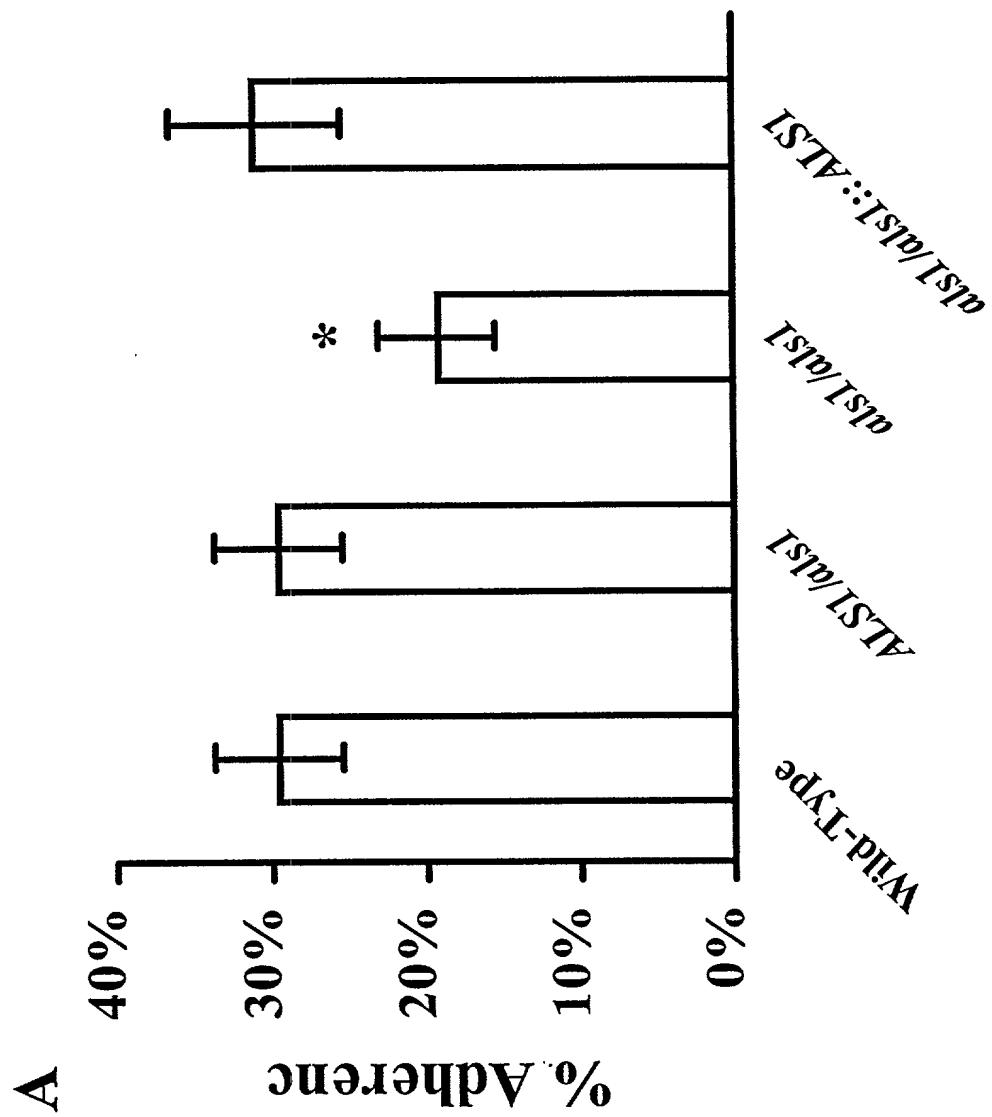
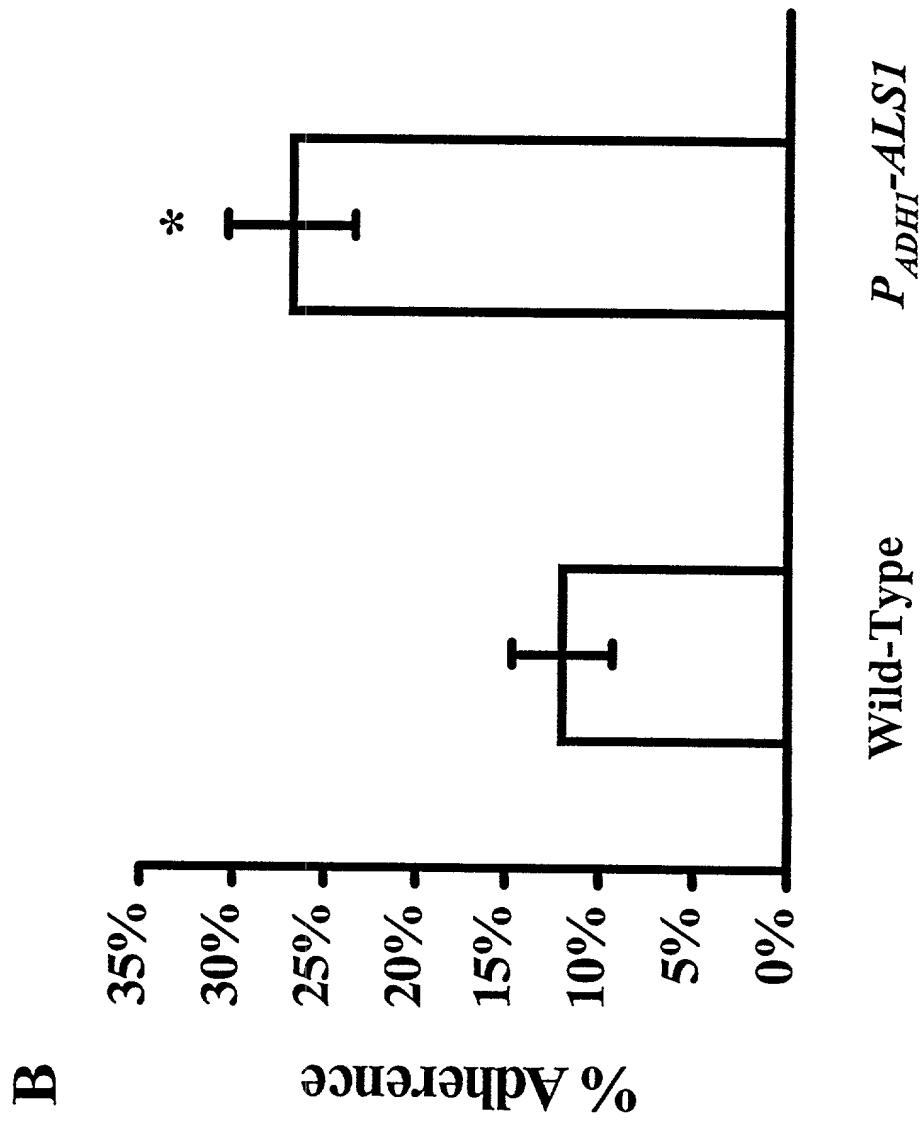
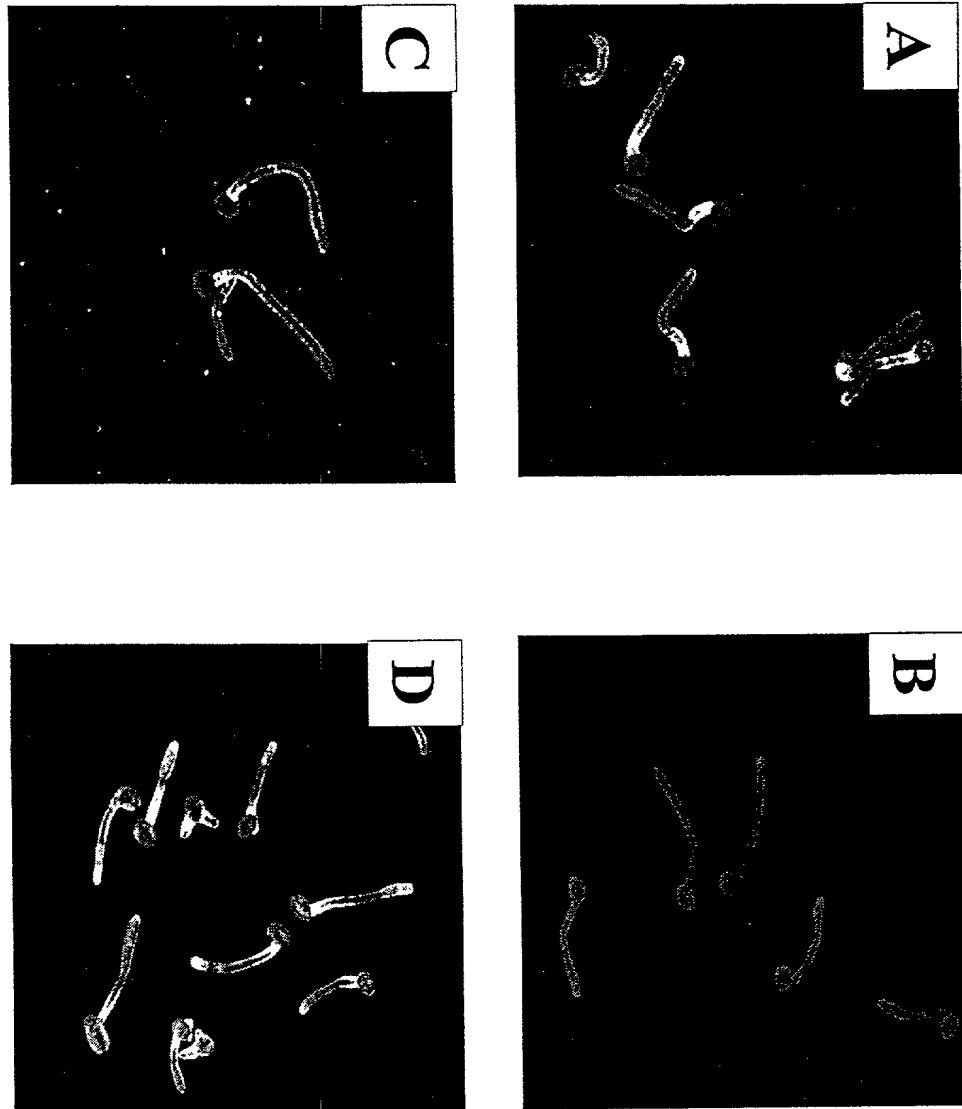


FIGURE 1B





**FIGURES 2A, 2B, 2C, 2D**

FIGURE 3A

A

Wild-Type

*als1/als1*

*als1/als1::ALS1*

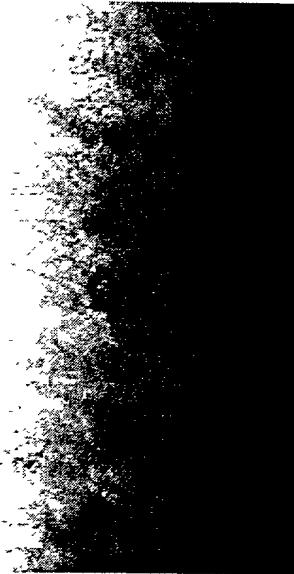
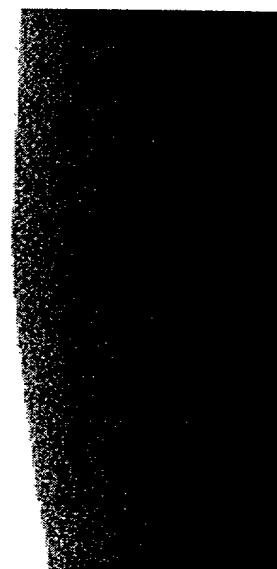
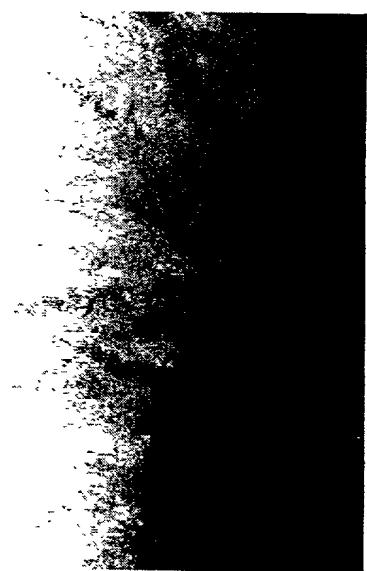
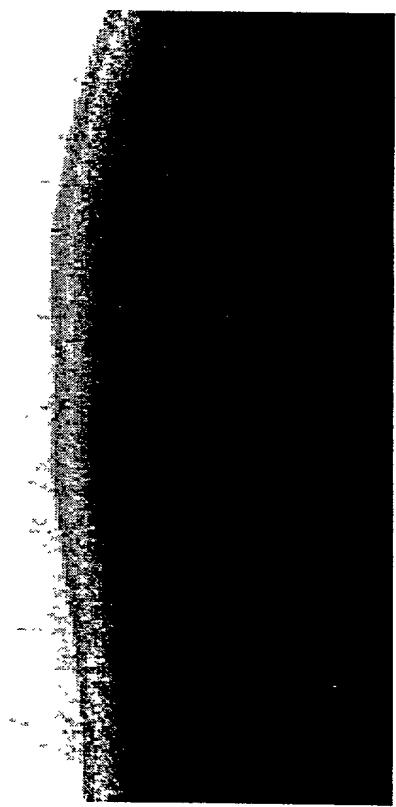
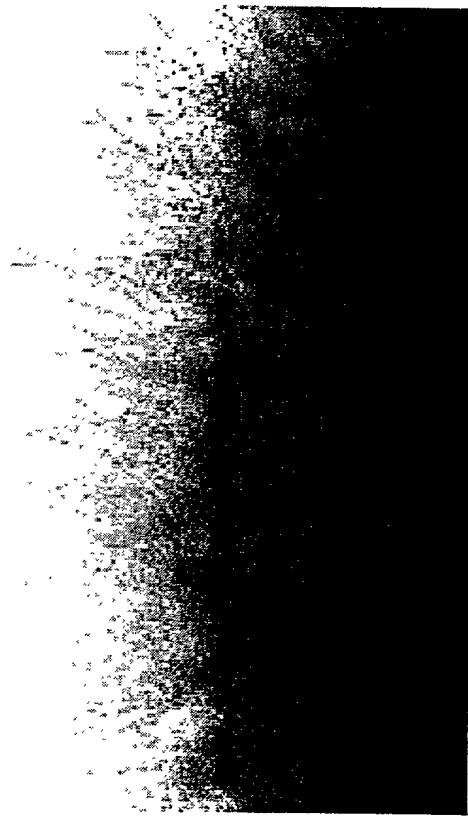


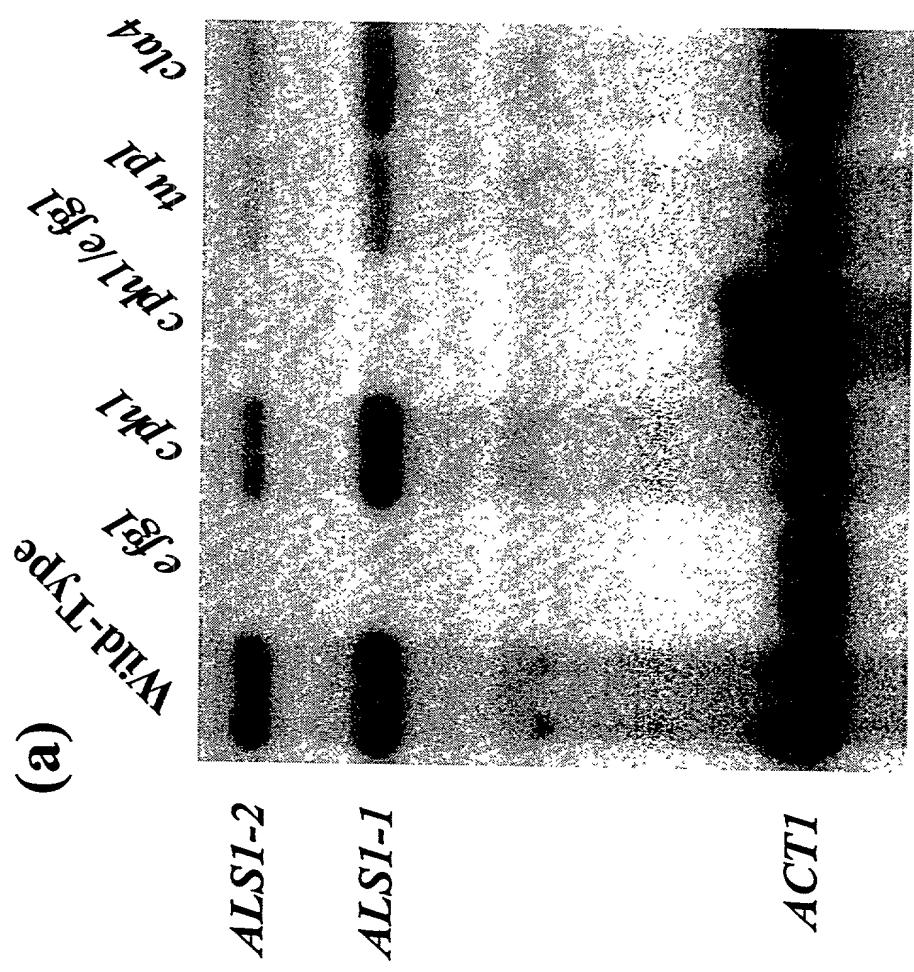
FIGURE 3B

**B**  
Wild-Type  
 $P_{ADH1-ALS1}$

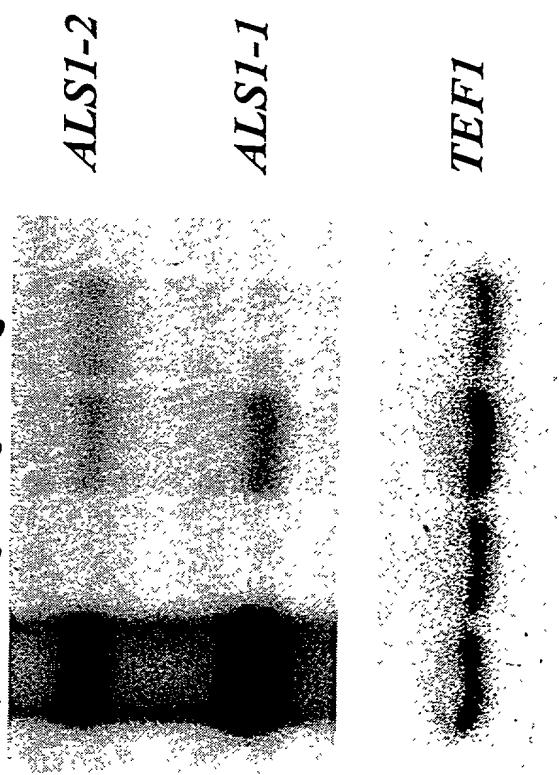


FIGURES 4A

A



(b)



*efg1/efg1*, *pADH1/ALS1*

*efg1/efg1/TEF1*

*efg1/efg1*

Wild-Type

FIGURE 4B

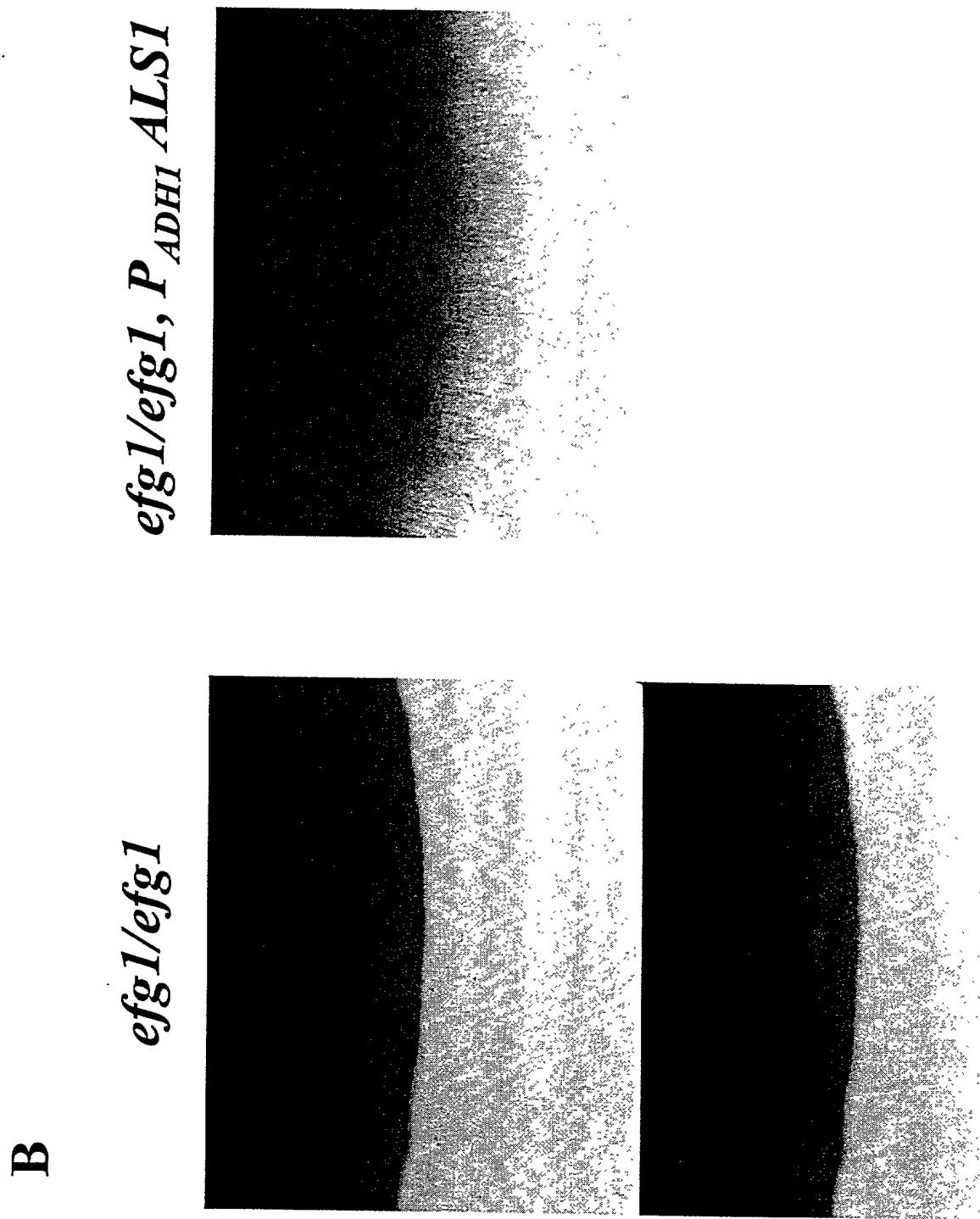


FIGURE 5A

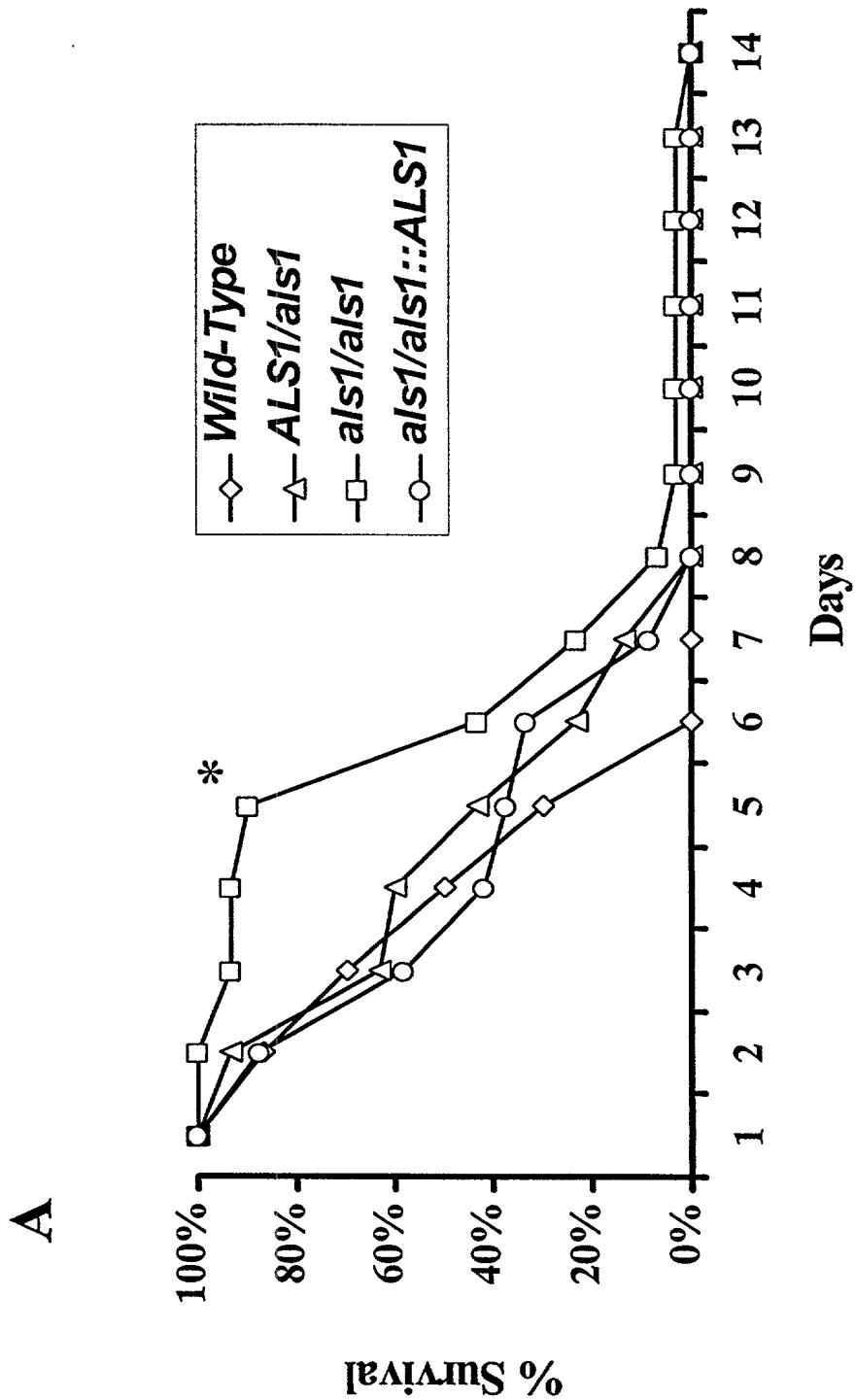


FIGURE 5B

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B

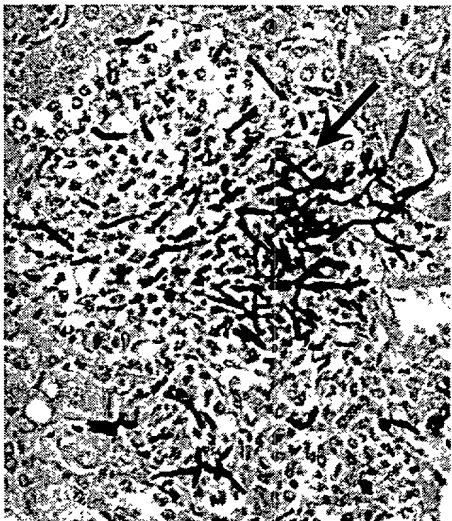
a Wild-Type



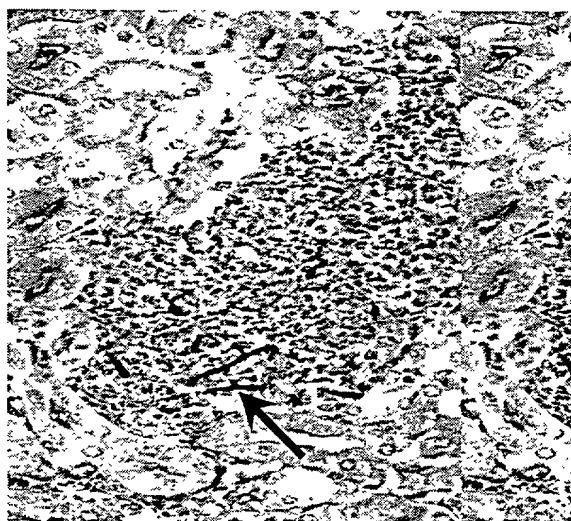
b Wild-Type



als1/als1::ALS1



als1/als1::ALS1



### Prophylactic effect of antiALS antibody against disseminated candidiasis

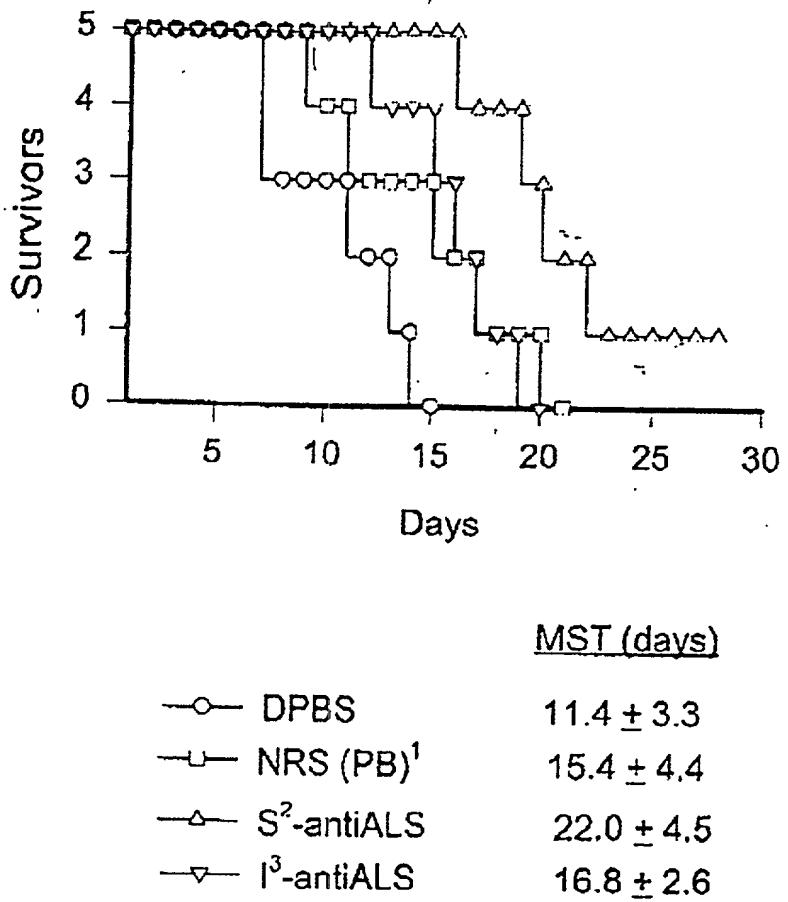


FIGURE 6

1 ATGCTTCAACAATTTACATTGTTATTCCATATTGTCAATTGCAAGTGCAAAGACAATC  
 1 M L Q Q F T L L F L Y L S I A S A K T I  
 61 ACTGGTGTGATAGTTAATTCAACTGGTCCAATGCTGCTAATTATGCTTTC  
 21 T G V F D S F N S L T W S N A A A N Y A F  
 121 AAAGGGCCAGGATAACCAACTTGGAAATGCTGTTGGTGGCCTTAGATGGTACCAAGT  
 41 K G P G Y P T W N A V L G W S L D G T S  
 181 GCCAATCCAGGGATAACATTACATTGAATATGCCATGTGTTAAATATACTACTTCA  
 61 A N P G D T F T L N M P C V F K Y T T S  
 241 CAAACATCTGTTGATTAAC TGCGATGGTGTAAATATGCTACTGTCAATTATTCT  
 81 Q T S V D L T A D G V K Y A T C Q F Y S  
 301 GGTGAAGAATTACAACCTTTCTACATTAACATGTACTGTGAACGACGCTTGAAATCA  
 101 G E E F T T F S T L T C T V N D A L K S  
 361 TCCATTAAGGCATTGGTACAGTTACCTTACCAATTGCATTCAATGTTGGTGGAACAGGT  
 121 S I K A F G T V T L P I A F N V G G T G  
 421 TCATCAAAC TGATTGGAAAGATTCTAAATGTTACTGCTGGTACCAATAACAGTCACATT  
 141 S S T D L E D S K C F T A G T N T V T F  
 481 AATGATGGTATAAGATATCTCAATTGATGTTGAGTTGAAAAGTCACCGTTGATCCA  
 161 N D G D K D I S I D V E F E K S T V D P  
 541 AGTGCATATTGTATGCTTCCAGAGTTATGCCAAGTCTCAATAAGGTACAACACTTTTT  
 181 S A Y L Y A S R V M P S L N K V T T L F  
 601 GTGGCACCAATGTGAAAATGGTTACACATCTGGTACAATGGGTTCTCCAGTAGTAAC  
 201 V A P Q C E N G Y T S G T M G F S S S N  
 661 GGTGACGTTGCTATTGATTGCTCAAATATTCAATTGGTATCACAAAAGGATTAAATGAT  
 221 G D V A I D C S N I H I G I T K G L N D  
 721 TGGATTATCCGGTTCATCTGAATCATTAGTTACACTAAAACCTGTACATCTAATGGA  
 241 W N Y P V S S E S F S Y T K T C T S N G  
 781 ATTCAGATTAAATATCAAATGTACCTGCTGGTTATCGTCCATTATTGATGCTTATATT  
 261 I Q I K Y Q N V P A G Y R P F I D A Y I  
 841 TCTGCTACAGATGTTAACCAATATACTTTAGCATACCAATGATTATACTTGCTGGC  
 281 S A T D V N Q Y T L A Y T N D Y T C A G  
 901 AGTCGTCTGCAAAGTAAACCTTCACTTAACATGGACTGGATACAAGAATAGTGATGCC  
 301 S R L Q S K P F T L R W T G Y K N S D A  
 961 GGATCTAACGGTATTGTCATTGCTACAACAGTACAGACAGTACCAACTGCT  
 321 G S N G I V I V A T T R T V T D S T T A  
 1021 GTCACTACTTACCAATTCAATCCAAGTGTGATAAAACCAAAACAATCGAAATTTGCAA

**FIGURE 7**

341 V T T L P F N P S V D K T K T I E I L Q  
 1081 CCTATTCCAACCACTACCACATCACAACTTCATATGTTGGTGTGACTACTTCCTATCTGACT  
 361 P I P T T T I T T S Y V G V T T S Y L T  
 1141 AAGACTGCACCAATTGGTGAACAGCAGCTACTGTTATTGTTGATGTGCCATATCATACTACC  
 381 K T A P I G E T A T V I V D V P Y H T T  
 1201 ACAACTGTTACCACTGAATGGACAGGAACAATCACTACCACCAACTCGTACCAATCCA  
 401 T T V T S E W T G T I T T T T R T N P  
 1261 ACTGATTCAATTGACACAGTGTTGACAAGTCCACTGCCAAATCCAACGTGTTAGTACT  
 421 T D S I D T V V V Q V P L P N P T V S T  
 1321 ACTGAATATTGGTCTCAGTCCTTGCTACAACCACTACAGTTACTGCTCCTCCAGGTGGT  
 441 T E Y W S Q S F A T T T V T A P P G G  
 1381 ACCGATACTGTGATTATCAGAGAGGCCACCAACCATACTGTCACTACTACTGAATATTGG  
 461 T D T V I I R E P P N H T V T T T E Y W  
 1441 TCACAATCCTTGCTACTACTACTGTTACTGCTCCTCCAGGTGGTACTGACTCAGTA  
 481 S Q S F A T T T V T A P P G G T D S V  
 1501 ATTATCAGAGAACCAACCAATCCAACGTCACTACAACCGAGTATTGGTCTCAATCCTT  
 501 I I R E P P N P T V T T T E Y W S Q S F  
 1561 GCTACTACTACAGTTACTGCTCCTCCAGGTGGTACTGACTCAGTAATTATCAGAGAA  
 521 A T T T V T A P P G G T D S V I I R E  
 1621 CCTCCAAACCCAACGTCAACCACCAACTGAATATTGGTCCAACTTACGCAACCAACT  
 541 P P N P T V T T E Y W S Q S Y A T T T  
 1681 ACTGTGACTGCTCCTCCAGGAGGCACTGACTCAGTAATTATCAGAGAACCAACCAAC  
 561 C T V T A P P G G T D S V I I R E P P N H  
 1741 ACTGTCACTACTGAATACTGGTCACAATCATATGCCACCACTACCACTGTAACGTCA  
 581 T V T T E Y W S Q S Y A T T T V T A  
 1801 CCACCAGGTGGTACTGACACTGTTATCATTAGAGAGGCCACCAACCAACTGTCACACT  
 601 P P G G T D T V I I R E P P N H T V T T  
 1861 ACTGAGTATTGGTCTCAATCGTTGCTACTACCACAACTGTAACGTGTCACCAAGTGGC  
 621 T E Y W S Q S F A T T T V T G P P S G  
 1921 ACTGATACTGTTATCATTAGGGAACCAACCAACCAACTGTCACCACTACTGAATACTGG  
 641 T D T V I I R E P P N P T V T T T E Y W  
 1981 TCTCAATCATATGCAACCACTACTACCATTACCGCTCCACCTGGTGAACGTGATACCGTT  
 661 S Q S Y A T T T I T A P P G E T D T V  
 2041 CTTATCAGAGAGGCCACCAACCAACTGTCACCACTACTGAATACTGGTCTCAATCATAT  
 681 L I R E P P N H T V T T E Y W S Q S Y  
 2101 GCTACAACCAACCACTGTTACTGCACCACTGGTGAACCGATACCGTTCTTATCAGAGAG  
 701 A T T T V T A P P G E T D T V L I R E  
 2161 CCACCAAACCAACTGTCACCACTACTGAATACTGGTCTCAATCATATGCTACAACCAAC

**FIGURE 7**

721 P P N H T V T T E Y W S Q S Y A T T T  
 2221 ACTGTTACTGCACCACCAAGGTGGTACCGATACTGTTATCATTAGAGAGCCACCAAATCCA  
 741 T V T A P P G G T D T V I I R E P P N P  
 2281 ACAGTTACTACTGAATATTGGTCACAATCATTGCCACAACCACACAGTTACTGCT  
 761 T V T T T E Y W S Q S F A T T T V T A  
 2341 CCTCCAGGTGGTACTGACACTGTGATTATCTATGAAAGCATGTCAAGTTCAAAGATTCT  
 781 P P G G T D T V I I Y E S M S S S K I S  
 2401 ACATCCTCCAATGATATAACCAAGTATCATTCCATCATTTCGGTCTCATTATGTCAAC  
 801 T S S N D I T S I I P S F S R P H Y V N  
 2461 AGCACAACTCCGATTGTCAACATTGAATCTTCATCCATGAATACTCCTACTTCTATC  
 821 S T T S D L S T F E S S S M N T P T S I  
 2521 AGTAGTGATGGTATGTTGTGTTCTACAACTTGGTACTGAATCAGAAACAACATA  
 841 S S D G M L L S S T T L V T E S E T T T  
 2581 GAACTGATTGCAGTGATGGTAAAGAGTGTCTAGATTGCCAGTTCTCTGGTATTGTC  
 861 E L I C S D G K E C S R L S S S S G I V  
 2641 ACAAAATCCAGATAGCAATGAATCCTCAATCGTAACTAGTACTGTTCTACTGCAAGTACA  
 881 T N P D S N E S S I V T S T V P T A S T  
 2701 ATGTCTGATTCACTTCTCAACTGATGGTATTAGTGCTACATCTCTGATAATGTTCA  
 901 M S D S L S S T D G I S A T S S D N V S  
 2761 AAATCAGGAGTATCAGTTACAACCGAAACTCTGTACAACATTCAAACACTCTCAAAC  
 921 K S G V S V T T E T S V T T I Q T T P N  
 2821 CCATTATCATCTTCAGTGACATCATTGACTCAGTTGTCTCAATTCCAAGTGTTCAGAA  
 941 P L S S S V T S L T Q L S S I P S V S E  
 2881 AGTGAAAGTAAAGTTACATTACAAGCAATGGAGACAACCAAGTGGTACTCATGATTCA  
 961 S E S K V T F T S N G D N Q S G T H D S  
 2941 CAATCTACTTCCACTGAAATTGAAATTGTAACAACCAAGTTCTACTAAAGTTTACCACT  
 981 Q S T S T E I E I V T T S S T K V L P P  
 3001 GTCGTTCTTCTAATACTGATTGACTAGTGAAACCAACAAATACCAAGAGAACCAACT  
 1001 V V S S N T D L T S E P T N T R E Q P T  
 3061 ACATTATCAACTACTTCAAACACTCCATCACTGAAGATATCACCAACATCTAACCTACAGGT  
 1021 T L S T T S N S I T E D I T T S Q P T G  
 3121 GATAATGGAGACAATACTTCATCAACCAATCCAGTTCAACTGTGGCAACAAGTACTTTA  
 1041 D N G D N T S S T N P V P T V A T S T L  
 3181 GCATCTGCAAGTGAAGAAGACAACAAAGCGGTTCTCATGAATCAGCATCCACAAGTTG  
 1061 A S A S E E D N K S G S H E S A S T S L  
 3241 AAACCAAGTATGGGTGAAAATTCTGGATTAACTACTTCTACTGAAATTGAAGCTACAACA  
 1081 K P S M G E N S G L T T S T E I E A T T  
 3301 ACCAGTCCTACAGAAGCTCCATCACCTGCTGTTCTGGTACTGATGTAACACTGAA

**FIGURE 7**

1101 T S P T E A P S P A V S S G T D V T T E  
3361 CCAACTGATACTAGAGAACAAACCTACTACATTATCAACTACTTCAAAAACAAACAGTGAA  
1121 P T D T R E Q P T T L S T T S K T N S E  
3421 CTGGTTGCTACTACACAAGCTACTAATGAAAATGGTGGTAAATCTCCATCAACTGATTAA  
1141 L V A T T Q A T N E N G G K S P S T D L  
3481 ACATCAAGCTTGACAACAGGCACCTCAGCATCTACAAGTGCTAATAGCGAACTTGTTACT  
1161 T S S L T T G T S A S T S A N S E L V T  
3541 AGTGGATCTGTTACTGGTGGAGCTGTTGCCAGTGCTTCAAATGATCAATCACATTCTACT  
1181 S G S V T G G A V A S A S N D Q S H S T  
3601 TCTGTTACCAACAGCAACAGCATTGTATCTAATACCCCACAAACTACATTGAGTCAACAA  
1201 S V T N S N S I V S N T P Q T T L S Q Q  
3661 GTTACCTCATCCTCACCTCAACCAACACATTGCTTCTACATACGATGGCTCTGGT  
1221 V T S S S P S T N T F I A S T Y D G S G  
3721 TCTATTATCCAACATTCTACTTGGTTGTACGGTTGATCACATTATTGTCCTGTTCATT  
1241 S I I Q H S T W L Y G L I T L L S L F I  
3781 TAGTGA  
1261 \* \*

FIGURE 7